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# TGF $\alpha$ expression in myeloid malignancies

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## ABSTRACT

**Background** Transforming growth factor  $\alpha$  (TGF $\alpha$ ) is a peptide growth factor known to be expressed in normal haemopoiesis. It is also expressed in a range of epithelial neoplasms but has not been assessed in haemopoietic malignancies. We have performed an immunohistochemical evaluation of TGF $\alpha$  in acute and chronic myeloid malignancies.

**Methods** TGF $\alpha$  expression was semiquantitatively assessed in 69 normal bone marrow trephines and 157 cases of myeloid malignancy using an immunohistochemical approach.

**Results** Blast cells of myeloid origin in acute myeloid leukaemia (AML), myelodysplasia and accelerated and blast phases of chronic myeloid leukaemia (CML) were TGF $\alpha$  positive. In acute promyelocytic leukaemia the neoplastic cells had significantly weaker TGF $\alpha$  expression than seen in other forms of AML. The blast cells in CML-accelerated and blast phases were positive with similar expression to AML.

**Conclusions** TGF $\alpha$  is expressed in neoplastic myeloblasts and could, therefore, be used as blast cell biomarker in diagnostic haematopathology. In addition, TGF $\alpha$  immunohistochemistry may be of use in identifying a therapeutic target.

## BACKGROUND

Transforming growth factor  $\alpha$  (TGF $\alpha$ ) is a member of the epidermal growth factor (EGF) family of peptide growth factors. Binding of TGF $\alpha$  to its cell surface ligand, EGF receptor (EGFR), results in receptor dimerisation and tyrosine kinase activity. This leads to activation of downstream signalling pathways, including Ras/Raf/MAPK and PI3K/Akt, resulting in cell proliferation and differentiation. TGF $\alpha$  is present in embryogenesis and is transiently expressed in some foetal tissues where it controls epidermal development. It is a recognised growth factor in chicken erythropoiesis, maintaining progenitor self-renewal, with withdrawal resulting in terminal erythroid differentiation.<sup>1</sup> In human bone marrow (BM) it is mainly expressed by erythroid progenitors.<sup>2</sup> TGF $\alpha$  is also reported to be expressed by promyelocytes, myelocytes, eosinophil precursors<sup>2</sup> and megakaryocytes.<sup>3</sup> It is not expressed by mature neutrophils or lymphoid cells in the BM, lymph node or spleen. TGF $\alpha$  expression is also found in multiple non-haemopoietic tissues, including epithelial cells, smooth muscle and brain, where it plays a physiological role in angiogenesis, wound healing and neural cell proliferation. TGF $\alpha$  plays a role in bone remodelling by inhibiting chondrogenesis<sup>4</sup> and stimulating bone resorption through effects on osteoclast development.<sup>5</sup>

In addition to its physiologic role, TGF $\alpha$  may play a role in a number of pathological processes.

Mutations in the *TGFA* gene, which encodes TGF $\alpha$ , have been implicated in some cases of non-syndromic orofacial clefts.<sup>6–7</sup> In mouse models TGF $\alpha$  signalling contributes to progressive pulmonary fibrosis through EGFR activation.<sup>8–9</sup> TGF $\alpha$  is expressed in several malignancies (eg, gastric carcinoma and melanoma) where TGF $\alpha$ –EGFR signalling may drive tumour growth via autocrine growth factor mechanisms.<sup>10</sup>

Despite the known expression of TGF $\alpha$  in normal myelopoiesis and that it is implicated in non-haemopoietic malignancy, there are no studies assessing TGF $\alpha$  expression in myeloid malignancies. In this study, we have assessed, using an immunohistochemical approach, the expression of TGF $\alpha$  in BM biopsies from patients with acute and chronic myeloid malignancies. Detection of TGF $\alpha$  in haemopoietic malignancies could have both diagnostic and therapeutic implications.

## METHODS

### Samples

BM trephine biopsy specimens from patients with acute myeloid leukaemia (AML), chronic myeloid leukaemia (CML), myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN) and non-malignant controls were selected for study. Table 1 lists the diagnostic subclassification and number of cases within each of these categories. The study had ethical approval and all patients had given consent for their BM to be studied.

### Immunohistochemical staining

BM specimens were sectioned at 4  $\mu$ m and placed on positively charged slides for immunohistochemical analysis on a BOND RX automated immunostainer (Leica Biosystems, Australia). Normal kidney was included in all staining experiments. Positive (renal tubule) and negative (interstitial cell) staining was clearly differentiated. Omission of primary antibody on a renal section was used as a negative control to exclude non-specific antibody binding. All slides were dewaxed in Bond dewax solution (product code AR9222, Leica Biosystems) before undergoing heat-induced epitope retrieval at pH 9 for 20 min (Bond epitope retrieval solution 2, product code AR9640, Leica Biosystems) during the automated staining process. TGF $\alpha$  antibody (clone 213-4.4, product code MA1-91686, Thermo Scientific, USA) was diluted 1:300 in Bond primary antibody diluent (product code AR9352, Leica Biosystems) and incubated on BM sections for 30 min. An alkaline phosphatase Bond Polymer Refine Red Detection kit (product code DS9390, Leica Biosystems) was used to detect antigen binding. Sections were counterstained in haematoxylin and mounted in Ultramount 4 mounting media (Hurst Scientific, Australia). CD61



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**Table 1** List of cases studied

Category	Subtype	Number
Non-malignant	Normal/reactive bone marrow	55
	Paget's disease of bone	7
	Renal osteodystrophy	7
AML	AML, not otherwise specified	21
	Acute promyelocytic leukaemia	9
Chronic myeloid leukaemia	Chronic phase	12
	Accelerated phase	12
	Blast phase	21
Myelodysplastic syndromes	Various WHO subtypes	13
Myeloproliferative neoplasms	Polycythaemia vera	7
	Essential thrombocythaemia	32
	Myelofibrosis	26
	Systemic mastocytosis	4
Total		226

AML, acute myeloid leukaemia.

(clone 2f2, product code NCL-CD61-308, Leica Biosystems), diluted 1:100, was used to assess megakaryocytes in some cases.

### Cytochemistry

Tartrate-resistant acid phosphatase (TRAP) staining was performed to identify osteoclasts in some BM sections. Dewaxed BM sections were incubated in 0.01% Naphthol AS-MX phosphate substrate in ethylene glycol monoethyl ether in buffer (100 mM acetate buffer, 50 mM sodium tartrate dihydrate, 0.22% glacial acetic acid and pH 5.0) for 1 h at 37°C, counterstained in haematoxylin and mounted in Ultramount Aqueous Permanent Mounting Medium (DAKO, Australia) for light microscopy.

### Microscopy

All stained sections were assessed by light microscopy using an Olympus BX53 microscope (Olympus Imaging Australia, Australia) and photographed using a Pixera Pro 600ES microscope camera (Pixera, USA). TGF $\alpha$  positivity in myeloid blasts was scored on a 0–3 point system where no staining scored 0, weak staining scored 1, moderate staining scored 2 and strong staining scored 3.

### Statistics

Student's two-tailed t test was used to evaluate differences in mean staining intensity between blasts in the different phases of CML and between AML/acute promyelocytic leukaemia (APML) cases.

## RESULTS

### Non-malignant disorders

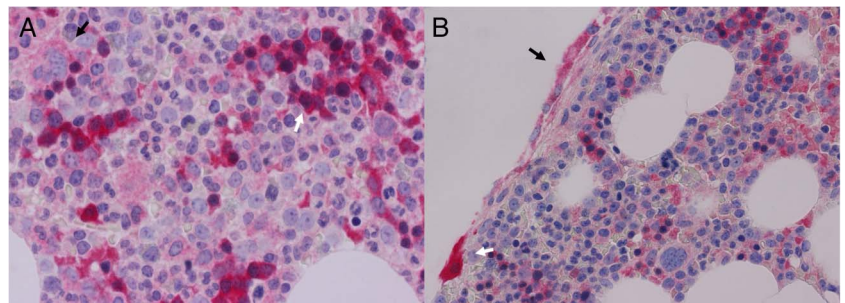
Good quality staining with clear distinction of positive and negatively stained cells was observed with the TGF $\alpha$  antibody in positive control tissue; negative controls showed no staining. In normal and reactive BMs TGF $\alpha$  staining was present in the erythroid lineage throughout differentiation, megakaryocytes, early granulopoiesis, osteoblasts and osteoclasts, in keeping with published literature (figure 1). In the erythroid lineage staining intensity increased to a maximum at the late normoblast stage. Anucleate erythrocytes were positive though less intense than the late normoblasts. Scattered blast cells in normal marrow biopsies were weakly positive but staining decreased throughout granulopoiesis; promyelocytes, myelocytes and mature granulocytes were negative. Osteoblasts and osteoclasts, present in small numbers in the normal and reactive marrows, were TGF $\alpha$  positive. We confirmed this reactivity in the cases of renal osteodystrophy and Paget's disease of bone. Osteoblasts were of moderate staining intensity whereas osteoclasts were intensely positive. All identified blood vessels demonstrated moderate intensity TGF $\alpha$  immunoreactivity in smooth muscle cells. Skeletal muscle, where present, demonstrated moderate intensity TGF $\alpha$  staining.

### Myeloid malignancies

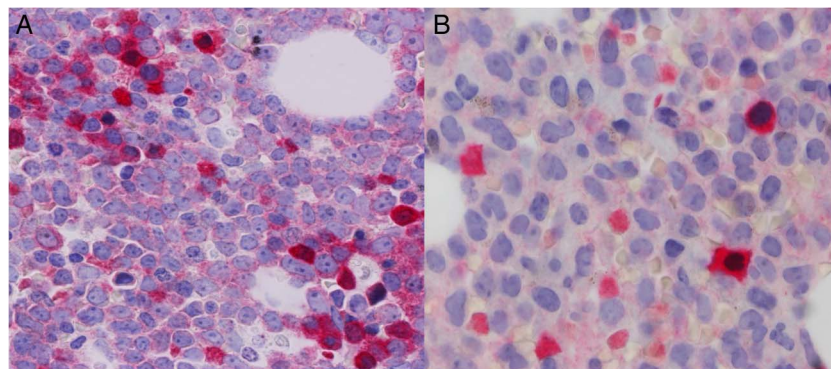
*Acute myeloid leukaemia:* leukaemic myeloblasts were TGF $\alpha$  positive in 28/30 (93%) cases of AML. The staining was weak in 10/28 (36%) and of moderate intensity in 18/28 (64%) cases. All blast cells were uniformly positive within a specimen (figure 2). Three of the 32 AML cases were erythroleukaemia (two erythroid/myeloid and one pure erythroid leukaemia); all demonstrated moderate intensity staining of the leukaemic blasts and strong staining of dispersed, maturing and erythroid precursors. Of the 30 AML cases, there were nine cases of APML. These showed overall weaker TGF $\alpha$  staining than other AML cases (figure 2) and two were negative. The mean staining intensity of the blasts in APML was 1.0 compared with 1.8 in the other AML cases ( $p=0.0004$ ; figure 3).

*Chronic myeloid leukaemia:* twelve cases of CML-chronic phase (CML-CP), all of which had <5% morphologically identifiable blast cells, were evaluated. The blast cells were weakly positive (10/12; 83%) or negative (2/12; 17%); maturing granulocytic cells were uniformly negative. In keeping with normal marrow, the megakaryocytes were weakly positive (figure 4). The blast cells, all previously demonstrated to be of myeloid origin, in CML-accelerated phase (CML-AP) and CML-blast phase (CML-BP) were universally TGF $\alpha$  positive. The mean blast staining intensity was 0.8 in CML-CP compared with 1.7 in CML-AP ( $p<0.0001$ ) and 1.8 in CML-BP ( $p<0.0001$ ; figure 3).

**Figure 1** Normal bone marrow stained for transforming growth factor  $\alpha$ . (A) Erythroid cells are moderately to strongly positive (white arrow) and megakaryocytes are weakly positive (black arrow). Magnification  $\times 600$ . (B) Intensely positive osteoclast adjacent to trabecular bone (white arrow) with moderate intensity staining in multiple osteoblasts (black arrow). Magnification  $\times 400$ .



**Figure 2** Cases of acute myeloid leukaemia stained for transforming growth factor  $\alpha$ . (A) Blast cells showing moderate intensity staining; residual erythroid precursors are strongly positive. Magnification  $\times 600$ . (B) Acute promyelocytic leukaemia showing weakly positive malignant promyelocytes. Magnification  $\times 600$ .



*Myelodysplastic syndromes:* erythroid cell staining was comparable in intensity to normal BM but the staining highlighted abnormally large or dispersed erythroid islands. Dysplastic megakaryocytes, confirmed by morphology and CD61 positivity, were weakly to moderately positive in 12/13 (92%) specimens. In one BM (refractory cytopenia with multilineage dysplasia) there were intensely TGF $\alpha$  positive interstitially located large cells with monolobated nuclei. These were confirmed to be dysplastic megakaryocytes by CD61 positivity and TRAP negativity. All cases with an identifiable blast component demonstrated weak staining of these cells; mature granulocytes were negative.

*Myeloproliferative neoplasms:* since TGF $\alpha$  was positive in normal megakaryopoiesis, we studied a large number of MPN cases ( $n=65$ ) with megakaryocytic hyperplasia to determine if there was any alteration in expression. No differences were seen: the megakaryocytes showed the same weak positivity for TGF $\alpha$

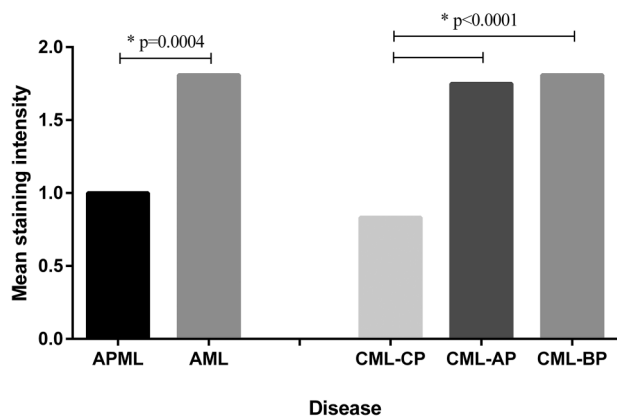
as seen in normal and reactive marrows, including immune thrombocytopenia. There was no association between staining intensity and MPN subtype, megakaryocyte size, location or morphology. In systemic mastocytosis ( $n=4$ ) the neoplastic spindle-shaped mast cells were weakly TGF $\alpha$  immunoreactive. Osteoblasts and osteoclasts were prominent with staining intensity in keeping with normal marrow.

## DISCUSSION

In this study, we have demonstrated that TGF $\alpha$  is expressed in early myeloid progenitors as evidenced by expression in normal and neoplastic myeloblasts. TGF $\alpha$  is retained through erythroid and megakaryocytic differentiation. In erythropoiesis the intensity of TGF $\alpha$  increases from early to late normoblastic differentiation whereas expression is unaltered throughout megakaryopoiesis.

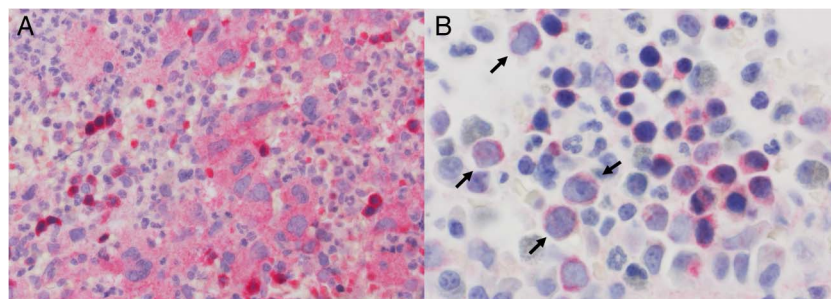
TGF $\alpha$  is expressed early in granulocytic differentiation and is lost following differentiation from the myeloblast to promyelocyte. Of greatest significance is that TGF $\alpha$  is expressed by neoplastic myeloid blasts. This was seen in all myeloid malignancies with a blast cell component, that is, AML, CML-AP, CML-BP and MDS. Significant differences were seen between APML and other types of AML. We propose that this reflects the greater degree of granulocytic differentiation in the malignant promyelocytes of this form of leukaemia. There were also significant differences in intensity of TGF $\alpha$  expression between the accelerated and blast phases of CML compared with the blast cells in chronic phase. Also of note is the fact that the cases of MDS in which there were an identifiable blast cell component demonstrated weak TGF $\alpha$  positivity, similar to that seen in normal BM specimens. Cases of AML generally demonstrated moderate-intensity TGF $\alpha$  positivity.

The precise cause for these findings is unclear from this study. While it is possible that TGF $\alpha$  plays a role in disease acceleration (eg, through previously suggested growth factor activity), other factors such as progressively diminished differentiation capability (and cytoplasmic accumulation of TGF $\alpha$ ) and



**Figure 3** Mean blast staining intensity showing differences between acute myeloid leukaemia (AML), acute promyelocytic leukaemia (APML) and the phases of chronic myeloid leukaemia (CML). AP, accelerated phase; BP, blast phase; CP, chronic phase.

**Figure 4** Cases of chronic myeloid leukaemia (CML) stained for transforming growth factor  $\alpha$ . (A) CML-chronic phase showing positive megakaryocytes and erythroid cells. Magnification  $\times 400$ . (B) CML-accelerated phase showing positively stained blast cells, arrowed. Magnification  $\times 1000$ .





differential activation of intracellular signalling pathways may all contribute to heightened TGF $\alpha$  staining in more acute leukaemia. Further work is required to clarify the precise mechanism/s underlying this change.

Since TGF $\alpha$  is highly expressed in myeloblasts, it may be a useful immunohistochemical blast cell 'biomarker' in BM specimens. In contrast to CD34, which is known to only be expressed in approximately 50% of AML cases,<sup>11</sup> TGF $\alpha$  was positive in 91% of AML cases we studied and showed uniform positivity throughout the leukaemic population. This makes it a robust marker with greater sensitivity than CD34 in BM specimens. Highlighting this was the finding that six cases of AML in our cohort were negative for both CD34 and CD117; TGF $\alpha$  was positive in all six (100%) of these cases. This demonstrates the potential of TGF $\alpha$  as a useful addition to the immunohistochemical repertoire for analysis of myeloid malignancies.

TGF $\alpha$  immunostaining could also be used to identify a potential immunotherapeutic target. Although specific TGF $\alpha$  inhibitors are not presently available for clinical use, several specific EGFR inhibitors are available. These agents have shown activity in cases of AML<sup>12–13</sup> as well as preventing or reversing pulmonary fibrosis in animal models.<sup>14</sup> This signalling axis may, therefore, be involved in the development of BM fibrosis.

In summary, TGF $\alpha$  is expressed in normal and neoplastic myeloblasts. These data have potential clinical application in both diagnostic capacity (as a blast cell 'biomarker') and as a potential new therapeutic approach. Further study is required to determine whether targeted therapy may play a role in targeted treatment of the myeloid disorders.

### Take home messages

- ▶ TGF $\alpha$  is expressed in multiple myeloid lineages; intensity of expression varies by lineage and degree of maturation.
- ▶ TGF $\alpha$  expression is significantly stronger in AML than APML and accelerated/blast phase CML than chronic phase CML.
- ▶ TGF $\alpha$  immunohistochemistry may be a useful addition to diagnostic haematopathology.

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obtained ethics approval for study and reviewed the manuscript. WNE undertook morphological review and reviewed the manuscript.

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